

## The Properties of Repressor and the Kinetics of its Action

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By experiments with mutants having temperature-sensitive repression systems for the *Lac* operon, it has been possible to provide evidence indicating that:

- (a) the *i*-gene product is the repressor, or physically included in it;
- (b) the rate of enzyme synthesis varies inversely with the first power of repressor concentration;
- (c) the repressor is "growth unstable" with a mean life of 1/5 to 1/10 generation;
- (d) inducers cause structural changes in repressor, manifested by altered thermal stability;
- (e) and the repressor is composed of subunits, which have a site for combination with inducers.

### 1. Introduction

The regulation of the synthesis of specific enzymes is believed to be mediated through the action of specific cytoplasmic substances, termed repressors, which act to damp the flow of information from structural gene to protein (Jacob & Monod, 1961). The repressor hypothesis as proposed by Jacob & Monod has been remarkably successful in accounting for the phenomena of inducible as well as repressible enzyme systems, and it has provided a productive framework for further experimentation. Nevertheless, doubt concerning its validity has originated in part from the paucity of direct experimental evidence concerning the repressor (Stent, 1964) and in part from observations on certain systems that apparently require a more complex explanation (Garen & Echols, 1962; Engelsberg, Irr & Power, 1964).

In the present work, we have been able to test some of the aspects of the repressor hypothesis and explore some of the properties of repressor through studies on the regulation of the synthesis of  $\beta$ -galactosidase in *Escherichia coli*. Here the evidence indicates that regulation of the "lactose" enzymes is mediated through a single species of macromolecule specified by the *i*-gene (Jacob & Monod, 1961; Willson, Perrin, Cohn, Jacob & Monod, 1964). The induction of  $\beta$ -galactosidase synthesis by certain galactosides, the inducers, shows a specificity only otherwise found in enzymes (Jacob & Monod, 1961; Willson *et al.*, 1964). Further, there is a mutation in the *i*-gene that leads to temperature-sensitive regulation of  $\beta$ -galactosidase synthesis (Horiuchi & Novick, 1961, 1965), which is attributable to the presence of a thermolabile repressor and, therefore, an indication that the repressor is a macromolecule.

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It was originally proposed that the repressor acts at the level of transcription of DNA into messenger RNA (Jacob & Monod, 1961). In agreement with this view, it has been found that the level of the *Lac*-specific m-RNA<sup>†</sup> changes upon induction (Hayashi, Spiegelman, Franklin & Luria, 1963; Attardi, Naono, Rouviere, Jacob & Gros, 1963; Guttman & Novick, 1963). However, the possibility remains that the primary site of action of repressor is elsewhere, the changes in messenger level being secondary consequences of this action.

Likewise, it is unknown whether the *i*-gene product acts *directly* upon some site on the DNA or m-RNA or *indirectly*, for example, through the production of yet another cytoplasmic constituent which in turn is the repressing agent. Also, earlier experiments could not distinguish whether the repressor turns over rapidly or whether it is stable, with the latter possibility requiring that rate of enzyme synthesis vary as a high power of level of repressor (Novick, Lennox & Jacob, 1963; Gallant & Stapleton, 1963).

In the present studies we have attempted to clarify some of these problems by using *i*-gene mutants exhibiting temperature-sensitive control of  $\beta$ -galactosidase synthesis to seek answers to the following questions.

(I) Is the *i*-product the repressor, rather than an enzyme needed for repressor synthesis?

(II) Does the differential rate of  $\beta$ -galactosidase synthesis vary inversely with the first power of level of repressor (R), or is the relationship of some higher order?

(III) Is the repressor a stable molecule?

(IV) Do inducers exert their effect directly upon the repressor, and are there alterations of the structure of repressor produced by inducer, as one might expect?

(V) What additional properties must the repressor have to account for the present results?

In this analysis, we make the assumption that repressor is a substance the effect of which is determined by its concentration in a bacterium. Further, we assume that the number of repressor molecules per cell is not usually so small as to produce significant heterogeneity in the bacterial population, an assumption which has been verified experimentally (Lennox, Novick & Jacob, 1963).

## 2. Materials and Methods

### *Nomenclature*

The term "temperature-sensitive" is applied to those mutant strains in which the regulation of  $\beta$ -galactosidase synthesis is defective at high growth temperatures but nearly normal at low temperatures. The term "thermolabile", abbreviated TL, is reserved to those strains which produce a heat-sensitive repressor for the *Lac* operon, while the phrase "temperature-sensitive synthesis" (TSS) is applied to those mutants in which synthesis of the specific *Lac* repressor, but not the repressor itself, is heat-sensitive.

*Organisms.* All experiments described were done with strains of *E. coli* K12 the properties of which are presented in Table 1. The original temperature-sensitive mutants are E103, E303 and E321. The derivation and properties of these are as follow.

(1) For E103:  $i^+ \rightarrow i^- \rightarrow i^{TL}$ . Isolated as an inducible "revertant" of a full constitutive E106 (Horiuchi & Novick, 1961, 1965), this allele is the result of at least two lesions in the *i* region. Its most distinctive property is the fact that repression of  $\beta$ -galactosidase synthesis can be destroyed by heating under conditions where growth is not possible,

<sup>†</sup> Abbreviations used: m-RNA, messenger RNA; TL, thermolabile; TSS, temperature-sensitive synthesis; IPTG, isopropylthiogalactoside.

TABLE 1  
*Characteristics and origins of bacterial strains*

Strain	Lac	Chromosomal			Episomal			Pro	B <sub>1</sub>	Met	Arg	Hist	Sm	Sex	Induced	Uninduced	Origin
E103	+	+TL	+	+				+	+	-	+	+	S	Hfr	1.4	0.050	Horiuchi <i>et al.</i> , 1962
E103a	-	+TL	+	-				+	+	-	+	+	S	Hfr	1.4	0.050	E103
E322	-	+TL	+	-				+	+	-	+	+	S	F-	1.3	0.035	E103
E325	-	+TL	+	-	+TL	+	-	+	+	-	+	+	S	F <sub>lac</sub>	2.7	0.023	E322 and E307
E303	-	s,TSS	+	+				+	-	+	-	-	S	F-	0.024	0.030	Novick <i>et al.</i> , 1963
E304	-	s,TSS	+	-				+	+	+	-	-	R	F-	0.024	0.030	E303
E310	-	s,TSS	+	-	s,TSS	+	-	+	-	+	-	-	R	F <sub>lac</sub>	0.003	0.004	E304 and E305
E305	-	+	+	+	s,TSS	+	+	+	-	+	-	-	S	F <sub>lac</sub>	0.03	0.003	F. Jacob
W14	-	+	+	-				-	-	+	+	+	R	F-	0.40	0.00047	Tomizawa, 1960
W14D	-	+	+	-	+	+	-	-	-	+	+	+	R	F <sub>lac</sub>	0.85	0.00048	W14 and E307
E321	-	+TSS	+	-				-	-	+	+	+	R	F-	0.50	0.020	W14
E323	-	+TSS	+	-	+TSS	+	-	-	-	+	+	+	R	F <sub>lac</sub>	1.0	0.0015	E321 and E307
W3747	+	+	+	+	+	+	+	+	+	-	+	+	S	F <sub>lac</sub>	1.8	0.001	S. E. Luria
E307	+	+	+	+	+	+	+	+	-	+	-	-	S	F <sub>lac</sub>	2.0	0.0005	F. Jacob

The column *Lac* refers to the ability to grow on lactose as sole carbon source at 37°C. *i*, *z*, *y* refer to the genotypes at these loci on the chromosome, and where present, on the modified sex factor *F*-*Lac*. *Pro*, *B*<sub>1</sub>, *Met*, *Arg* and *Hist* refer to the ability to synthesize proline, thiamin, methionine, arginine or histidine. *Sm* refers to sensitivity (S) or resistance (R) to streptomycin. Sex indicates mating type. Induced and Uninduced indicate the steady-state specific activity of  $\beta$ -galactosidase during growth at 37°C in the presence or absence of  $10^{-3}$  M IPTG. Origin notes the source or derivation of each strain.

the degree of de-repression obtained being a function of the time and temperature of heating. Under optimal conditions, de-repression is equivalent to optimal induction with IPTG.

(2) For E303:  $i^+ \rightarrow i^s \rightarrow i^{s,TSS}$ . Isolated as a  $Lac^+$  revertant of an  $i^s$  strain at 42°C (Willson *et al.*, 1964), this allele is also the result of at least two mutations in the  $i$ -gene. It retains the  $i^s$  character at all temperatures of growth, in that no augmentation in the rate of  $\beta$ -galactosidase synthesis over that produced by thermal de-repression is produced by the addition of IPTG. This allele differs from that in E103, since heating in the absence of growth has *no effect* on repression of  $\beta$ -galactosidase synthesis. All tests devised to demonstrate thermolability of repressor in this type have been negative. Instead, a number of observations indicate that some precursor of the finished repressor is heat-sensitive.

(3) For E321:  $i^+ \rightarrow i^{TSS}$ . Its properties are strikingly similar to those of E303, if not indistinguishable (see Figs 1 and 5), with the exception that E321 is inducible by IPTG.

The steady-state differential rates of  $\beta$ -galactosidase synthesis in the presence or absence of  $10^{-3}$  M-IPTG at various growth temperatures are shown for strains E103, E304, and E321 in Fig. 1.

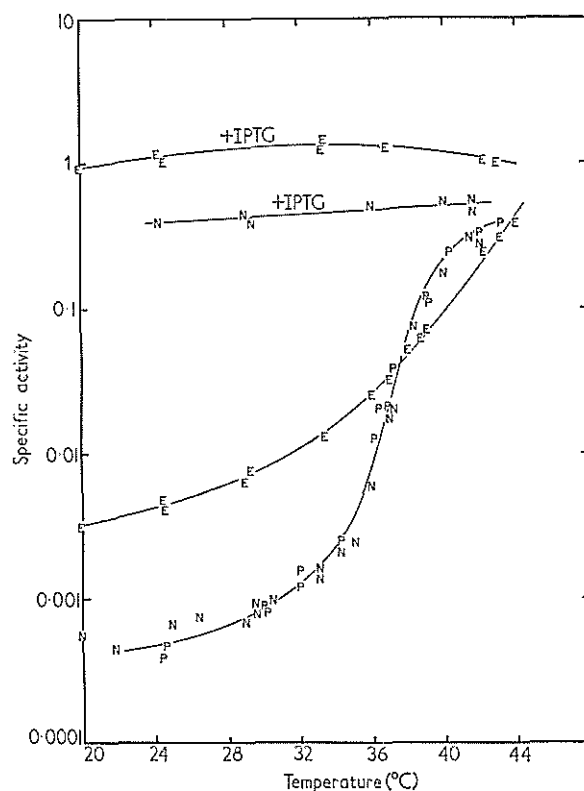


FIG. 1. Steady-state specific activities of  $\beta$ -galactosidase in the presence or absence of  $10^{-3}$  M-IPTG in the strains E103 ( $i^{TL}$ ), E304 ( $i^{s.TSS}$ ) and E321 ( $i^{TSS}$ ) during growth at various temperatures.

The letters E, P and N refer to the strains carrying the  $i$ -gene alleles  $i^{TL}$ ,  $i^{s.TSS}$  and  $i^{TSS}$ , respectively. Each point is the average of duplicate assays. The organisms were grown at the specified temperature for at least 10 doublings prior to sampling for assay. The medium in all cases was F buffer + 0.2% glycerol, supplemented with L-methionine (20  $\mu$ g/ml.) in the case of E103; L-proline (50  $\mu$ g/ml.) and thiamin (10  $\mu$ g/ml.) in the case of E321; and L-histidine (20  $\mu$ g/ml.), L-arginine (20  $\mu$ g/ml.) and thiamin (10  $\mu$ g/ml.) in the case of E304.

*Media*

Except where noted, all experiments were performed on cells growing in a liquid medium consisting of M/30 potassium phosphate buffer (pH 7), M/50 ammonium chloride,  $10^{-3}$  M-MgSO<sub>4</sub> plus 0.2% (v/v) glycerol (F buffer plus glycerol) supplemented where necessary with amino acids or vitamins. Matings were performed in Difco Pennassay broth. The phage  $f_2$  was propagated on cells growing in LB broth (Luria, Adams & Ting, 1960). Among the solid media used were Difco triphenyl-tetrazolium chloride-Tryptone broth-sugar (TTC), eosin-methylene blue-broth-sugar agar (EMB), and Davis minimal agar; these have been described earlier (Horiuchi, Tomizawa & Novick, 1962). When bacteria were grown in the chemostat, either nitrogen or carbon was used as the limiting nutrient, and the basic F-glycerol medium was modified accordingly. Thus, the concentration of NH<sub>4</sub>Cl in the reservoir was reduced to 15 mg/l. to give a steady-state population of about  $7 \times 10^7$  cells/ml. in the chemostat growth tube. Similarly, glycerol at 90 mg/l. supported a population of  $9 \times 10^7$  cells/ml. in the chemostat.

*Mutagenesis*

The mutagen N-methyl-N<sup>1</sup>-nitro-N-nitroso-guanidine (nitroso-guanidine), from the Aldrich Chemical Co., 2639 N. 29th Street, Milwaukee 10, Wisc., was used in the preparation of mutants such as E103a and E321. The parent organism was suspended at a density of 1 to  $3 \times 10^9$  cells/ml. in sterile 0.1 M-phosphate buffer, pH 6.3, containing freshly dissolved nitrosoguanidine at a final concentration of 5 to 10  $\mu$ g/ml. The suspension of cells was incubated at 37°C for 3 to 5 hr, washed, resuspended in sterile diluent and plated. From 10 to 70% of the cells normally survived this treatment.

*Selection of E322*

The phage  $f_2$  (obtained from N. Groman), which attacks only male strains of *E. coli* K12 (Zinder, 1961), was used to select E322 (F<sup>-</sup>) from E103a (Hfr). E103a growing in LB broth at  $5 \times 10^8$  cells/ml. (37°C) was infected with  $f_2$  previously propagated on E103a at a multiplicity of 20. After lysis and overgrowth of resistant variants (12 hr of incubation subsequent to infection), the culture was streaked out, incubated at 37°C, and 10 resistant clones picked, freed of phage, and tested for their mating type. None of the 10 clones transferred the *Lac* region with measurable frequency to a standard F<sup>-</sup> strain (E64N<sub>3</sub>), but in a mating with a standard *F-Lac* genote (E320) only 2 behaved as normal F<sup>-</sup> recipients. One of these was reserved and given the designation E322. Except for mating type, it is identical to E103a with respect to all properties tested (nutritional requirements and the genotype at the loci *i*, *z* and *y*).

*Selection of E321*

It was noted that the EMB-lactose agar reaction of many  $i^+z^+y^-$  strains changes from translucent purple to opaque white when IPTG ( $10^{-3}$  M) is included in the medium. It was reasoned that constitutive variants of these  $z^+y^-$  strains would give opaque white colonies in the absence of IPTG and could thus be easily distinguished from both the parental type  $i^+z^+y^-$  and permease-positive revertants ( $i^+z^+y^+$ ). This method was tested on nitrosoguanidine-treated cultures of W14 and E312. Some 12,000 viable cells from each strain were plated on EMB-lactose agar plates (about 300 per plate) and incubated for 18 hr at 42°C. A total of 88 opaque white colonies or sectors were picked, purified, and tested for the constitutive synthesis of  $\beta$ -galactosidase at 30 and 42°C. Of the 88 clones, 26 were found to synthesize  $\beta$ -galactosidase constitutively at 42°C and one of these, termed E321, derived from W14, exhibited a 500-fold increase in the rate of synthesis between 30 and 42°C.

The assumption that the mutation in E321 lies in the *i*-gene is based on the following observations.

(1) There is a close similarity in thermal properties to E303, in which the mutation is believed to lie in the *i*-gene (Willson *et al.*, 1964).

(2) The  $i^{TSS}$  allele is recessive to the  $i^+$  allele, i.e., *F-Lac* heterogenotes carrying  $i^{TSS}$  and  $i^+$  are not constitutive at high growth temperatures. Further,  $i^{TSS}/i^-$  as well as  $i^{s.TSS}/i^-$  diploids show the TSS phenotype; and  $i^{TSS}/i^{TL}$  as well as  $i^{s.TSS}/i^{TL}$  diploids are de-repressed at high temperatures.

*Preparation of F-Lac homogenotes*

Cultures of *F-Lac* heterogenotes of genotype  $z^+y^-/Fz^+y^+$  or  $z^-y^+/Fz^+y^+$  normally contain 0.1 to 1.0% *Lac*<sup>-</sup> recombinant segregants. The great majority of these prove to be *F-Lac* homogenotes of genotype  $z^+y^-/Fz^+y^-$  and  $z^-y^+/Fz^-y^+$ , respectively. The close linkage of the *i* to *z* and *y* suggests that the great majority of such *F-Lac* homogenotes would be homodiploid for the *i*-character from the chromosome alone, and this indeed proved to be the case among *Lac*<sup>-</sup> recombinants isolated from the *F-Lac* heterogenote  $i^s.tssz^+y^-/Fz^+y^+$ . This finding was used as the basis for the isolation of *F-Lac* homogenotes from the *F*<sup>-</sup> strains E322, E304, E321, W14 and E312. The resultant *F-Lac* homogenotes were tested for the character of *i*, *z* and *y* by mating with appropriate *F*<sup>-</sup> recipients and by examination of the recombinant types obtained, and also by physiological testing to ascertain both the number of *z* genes and the thermal properties of the regulation of  $\beta$ -galactosidase synthesis.

*Culture methods*

The concentration of bacteria was determined in a spectrophotometer at 350 m $\mu$ . Comparison of absorbancy measurements with plate counts and dry-weight determinations established that an absorbancy of 0.100 corresponded to  $3 \times 10^7$  cells/ml. or to 10  $\mu$ g dry weight of cells/ml. for bacteria growing in F-glycerol medium. Steady-state levels of  $\beta$ -galactosidase were measured only after the bacteria had grown at the stated temperature ( $\pm 0.1^\circ\text{C}$ ) in F-glycerol medium for at least 10 doublings. In temperature shifts involving batch cultures or chemostats, temperatures reached very nearly their ultimate values within 3 to 4 min.

*Transfer of cells to new media*

The rapid transfer of cells from a growth medium or buffer to a new growth medium was accomplished through the use of 45-mm membrane filters (0.45  $\mu$  pore diameter). The total time required was 2 to 5 min; from  $1$  to  $3 \times 10^9$  cells per filter was the optimum number both for rapid filtration and good recovery. Cells were washed on the filter 5 to 7 times by the addition of 100-ml. portions of F-buffer at the appropriate temperature and then resuspended in the new medium previously equilibrated at the proper temperature. To remove suspended material that might interfere with subsequent absorbance measurements, the wash buffer and the new medium were themselves filtered prior to use. To obtain immediate exponential growth of the filtered, resuspended cells, it was found necessary to use a medium preconditioned by growth of the same organism to about  $2 \times 10^8$  cells/ml. This medium was filtered free of cells, supplemented where necessary with additional amounts of glycerol and amino acids and stored at  $4^\circ\text{C}$  until use. Where it was important that all operations be carried out at high temperatures, the procedure was carried out in a warm room at  $38$  to  $40^\circ\text{C}$  with glassware and media equilibrated at that temperature.

*Enzyme assays*

$\beta$ -Galactosidase was determined in toluene-treated 1.0-ml. portions of bacterial cultures, as previously described (Horiuchi *et al.*, 1962). Color change was measured in a total volume of 4 ml., using a cuvette of 1-cm light path, and enzyme activities are given in  $\Delta\text{O.D.}_{420} \times 1000/\text{min}$ . Division by the bacterial density, taken as  $\text{O.D.}_{350} \times 1000$ , gives the specific activity; this is a convenient scale, since wild-type strains give fully induced activities near unity.

The galactoside permease was assayed through measurement of the extent of intracellular accumulation of  $\beta$ -methyl- $^{14}\text{C}$ -thiogalactoside by non-growing metabolizing cells (Kepes, 1960).

### 3. Results

#### I. Evidence that the *i*-product is the repressor

The repressor postulated by Jacob & Monod (1961) is a single molecular species with two functional sites, one which interacts with inducer and one which affects the operator, the point in protein synthesis where repression is established. The

existence of mutant *i*-genes having altered specificity toward inducers indicates that the *i*-product does interact with inducer (Willson *et al.*, 1964). In the present work, evidence has been found indicating that the *i*-product is also the agent directly responsible for repression; and in this sense it can be concluded that the *i*-product, having both functions, is the repressor as postulated.

Evidence pointing to the *i*-product as the repressing substance was obtained by observing the effect on rate of  $\beta$ -galactosidase synthesis of abrupt removal of the *i*-product. Such removal was achieved by a brief heating of a growing culture of a bacterial strain having an *i*<sup>TL</sup> allele. As can be seen in Fig. 2, enzyme synthesis reaches

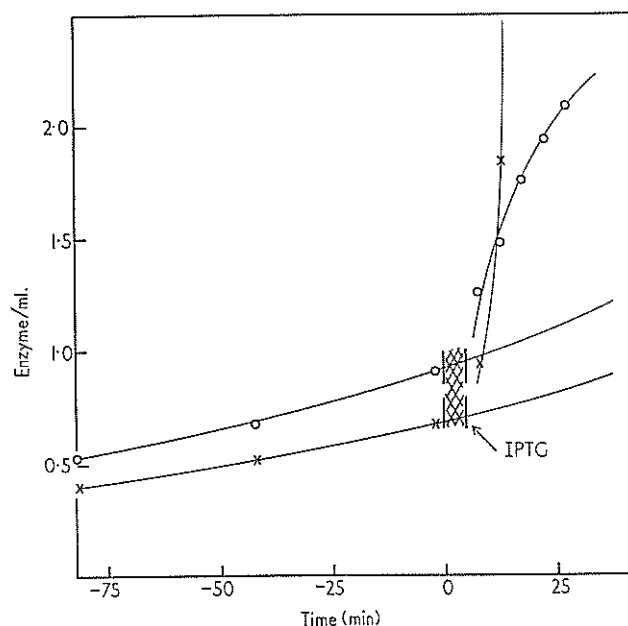


FIG. 2. Demonstration that de-repression is immediate following removal of the *i*-gene product by thermal inactivation.

Parallel cultures of E103 in minimal medium growing at 25°C were heated at 44°C for 7 min, starting at  $t = 0$ , indicated by the cross-hatched area, and then returned to 25°C for further growth. To one (—×—×—), IPTG was added to a final concentration of  $5 \times 10^{-4}$  M at  $t = 7$  min, while the other received no IPTG (—○—○—).

its maximum rate in a very short time following heating, a time not longer than that observed in a comparison culture de-repressed by the addition of IPTG. The immediate effect of *i*-product removal must mean that the *i*-product is the repressing agent (or an essential component). Were the *i*-product an enzyme involved in repressor formation, one would have to make the additional assumption that the repressor turns over very rapidly, with a lifetime of less than a minute.

## II. Dependence of rate of synthesis of enzyme on concentration of repressor

Earlier studies sought to determine the relationship between the rate of synthesis of  $\beta$ -galactosidase and the relative level of repressor by observing the rising rate of synthesis that occurs when repressor formation is arrested by the transfer of an

$i^{s,TSS}$  culture from low to high growth temperature (Novick *et al.*, 1963). Here de-repression occurs relatively rapidly, and it was not possible from such experiments alone to distinguish between two possibilities:

- (1) that the repressor is stable and repression varies as a high power of repressor level; or
- (2) that the repressor is unstable with a mean life of about 1/10 to 1/5 of a generation, and the dependence of the rate of enzyme synthesis is inverse with the first power of repressor concentration, as originally suggested in the case of similar experiments with alkaline phosphatase mutants (Gallant & Stapleton, 1963).

In order to distinguish between these two possibilities, an independent means for varying the level of repressor was developed here. This method is based on the assumption that a relative increase in number of  $i$ -genes in a bacterium gives a corresponding increase in repressor. For example, in an F-genote of type  $i^+z^+y^-/Fi^+z^+y^-$ , the level of  $\beta$ -galactosidase upon full induction in the F-genote is about 2.5 times that of the haploid  $i^+z^+$ . The F-genote is presumably diploid for the  $Lac$  genes, and the ratio being larger than two is attributed to the non-synchronous division of the chromosomal and episomal  $Lac$  genes (Jacob, personal communication). A similar gene dosage effect should exist for the  $i$ -gene, unless its expression is dependent on the level of repressor, this being rendered improbable in another study (Novick, McCoy & Sadler, 1965). Thus the repressor level in the F-genote,  $i^+/Fi^+$ , should be 2.5 times that in the haploid,  $i^+$ . Likewise it is reasonable to assume a similar ratio for  $i^{TL}$  and  $i^{TL}/Fi^{TL}$ , since the fraction of repressor thermally inactivated at any temperature should be independent of the concentration of repressor. It is not clear, however, whether the levels of repressor would be proportional to gene dosage for  $i^{TSS}$  and  $i^{s,TSS}$  alleles, where the rate of formation of repressor is temperature-dependent. The most plausible explanation for temperature-sensitive synthesis is the existence of a heat-labile precursor of the repressor in these mutants. Were the precursor a monomer which must be polymerized, it is evident that the level of repressor in the diploid could be much greater than 2.5 times that in the haploid. (It should be noted that the formation of recombinant segregants which prevent such analysis with heterogenotes of type  $i^+/i^-$ , for example, is no cause for concern with the homogenotes used here.)

To see the effect of such a change in repressor level, one must make comparisons where the rate of enzyme production,  $dz/dB$ , is a function of repressor level ( $R$ ), e.g. for temperature mutants at intermediate temperatures. The relative rates to be expected are shown in Table 2, where comparisons are made on the alternative hypotheses that the relationship is inverse with the first power of repressor ( $n = 1$ ) or that it is inverse with a high power of repressor. For simplicity, the comparison is made at low rates of synthesis where the indicated approximation can be made. It is assumed as argued above that the concentration of repressor in the "diploid" is 2.5 times that in the haploid.

It should be noted that there is a sharp difference in the expectations of the two hypotheses. For  $n = 1$ , the ratio of specific activities for haploid/diploid is one, whereas for  $n = 5$  it is close to 40.

This test was first applied to strains bearing the  $i^{TL}$  allele, and the rates of synthesis of  $\beta$ -galactosidase observed for the haploid  $i^{TL}$  and the diploid  $i^{TL}/Fi^{TL}$  as a function of temperature are shown in Fig. 3. It is evident that the haploid/diploid ratio is at most 1.5, indicating that  $n$  cannot be more than 2 (for which the ratio would be 2.5)



TABLE 2

Hypothesis	Expected rate of synthesis		Ratio of rates
	Haploid	Diploid	Haploid/Diploid
$\frac{dz}{dB} = \frac{K}{K+R} \sim \frac{K}{R}$	$\frac{K}{R_h}$	$2.5 \frac{K}{2.5 R_h}$	1
$\frac{dz}{dB} = \frac{K}{K+R^n} \sim \frac{K}{R^n}$	$\frac{K}{R_h^n}$	$2.5 \frac{K}{(2.5 R_h)^n}$	$2.5^{n-1}$

$\frac{dz}{dB}$  = rate of enzyme synthesis per cell = specific activity in steady state.

$R_h$  = repressor level in haploid.

$K$  = constant.

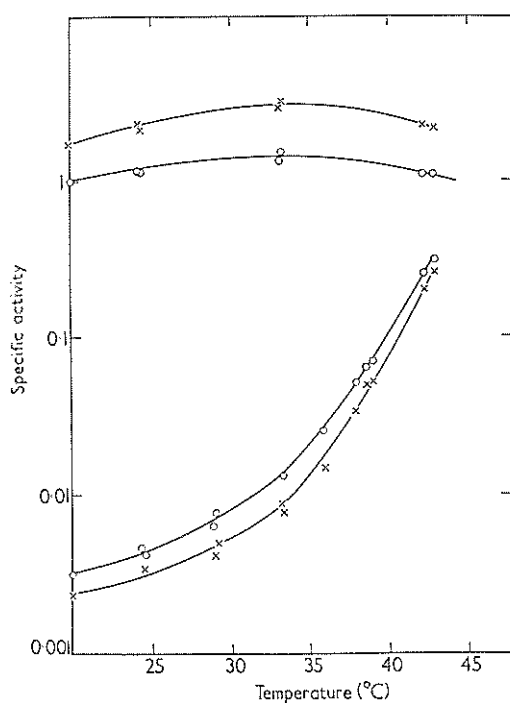


FIG. 3

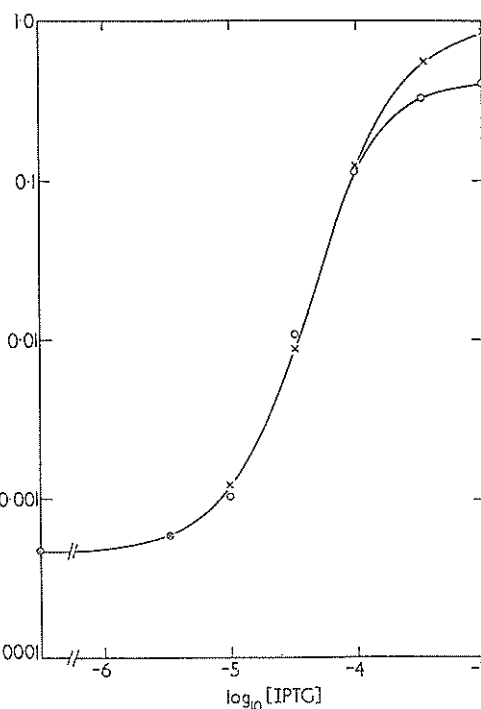


FIG. 4

FIG. 3. Steady-state specific activities of  $\beta$ -galactosidase in the presence or absence of  $10^{-3}$  M-IPTG at various growth temperatures for the strains E322 and E325.

Parallel cultures of E322 ( $i^{TL}z^{+}y^{-}$ ) and E325 ( $i^{TL}z^{+}y^{-}/Fi^{TL}z^{+}y^{-}$ ) were grown at the specified temperature for at least 10 doublings prior to sampling for assay. The minimal medium used was the same as that specified for E103 in Fig. 1. The upper curves were obtained in the presence of  $10^{-3}$  M-IPTG. Each point for E322 ( $-O-O-$ ) and E325 ( $-X-X-$ ) is the average of duplicate assays.

FIG. 4. Steady-state specific activities of  $\beta$ -galactosidase for W14 and W14D growing at  $37^{\circ}\text{C}$  in the presence of various concentrations of IPTG.

W14 ( $i^{+}z^{+}y^{-}$ ) and the derivative  $F$ -Lac homogenote W14D ( $i^{+}z^{+}y^{-}/Fi^{+}z^{+}y^{-}$ ) were grown in the specified concentration of IPTG for at least 10 doublings prior to sampling for assay. The medium used was the same as that specified for E321 in Fig. 1. Each point for haploid ( $-O-O-$ ) and  $F$ -Lac homogenote ( $-X-X-$ ) is the average of duplicate assays on each of three independent cultures.

and is most probably 1. The slight departure from unity in the ratio would result if the relative rates of formation of repressor were even very slightly dependent on gene dose, as was considered above for the  $i^{TSS}$  alleles.

A similar result was obtained in a comparison of  $i^+z^+y^-$  haploid (W14) with an  $i^+z^+y^-/F i^+z^+y^-$  diploid (W14D) (see Fig. 4). Here the comparison is made at a series of concentrations of inducer, making the assumption that inducer causes an effective reduction in concentration of repressor, a reduction which is independent of level of repressor. Several other independent pairs of  $i^+z^+y^-$  haploids and derivative homogenotes were tested with identical results.

A like comparison (rate of enzyme synthesis *versus* IPTG concentration) was made for  $i^{TL}z^+y^-$  *versus*  $i^{TL}z^+y^-/F i^{TL}z^+y^-$  at low growth temperatures (about 20°C), where repression is very nearly normal, with substantially the same results as those shown in Fig. 4.

Further evidence in support of the conclusion that  $dz/dB$  varies as  $1/R$  comes from experiments (described in section IV of Results) in which it was found that small amounts of repressor produce a sharp reduction in  $dz/dB$  in cells initially lacking repressor. Such a reduction would not be expected if there were a high power dependence.

### (III) The "growth-instability" of repressor

As noted earlier, the first evidence suggesting that repressor is unstable was the unexpected rapid rise in the rate of enzyme synthesis following arrest of repressor formation in  $i^{TSS}$  mutants. An illustration of such a rise is given in Fig. 5, where repressor synthesis was stopped in both  $i^{s.TSS}$  and  $i^{TSS}$  strains by a shift in growth temperature from 30 to 41.5°C. The possibility that there is a thermal inactivation of repressor is discounted by the observation that an  $i^{TSS}$  mutant, grown at 30°C and then heated at 42°C for two hours in the absence of growth (no carbon source), showed no appreciable de-repression upon further growth at 30°C. Further demonstration that thermolability plays no significant part in the repressor instability of  $i^{TSS}$  mutants is given in Fig. 6. Here are shown the results of temperature shifts (30 to 41.5°C) of an  $i^{s.TSS}$  mutant growing glycerol-limited at a number of widely different rates in the chemostat. Were the repressor appreciably thermolabile, de-repression would be relatively much more rapid in cultures growing slowly (15-hour generation time) than in those growing with a generation time of two to four hours. Moreover, the rate of de-repression following the shift to growth at high temperature is independent of the value of the higher temperature (Novick *et al.*, 1963). Evidently the rate of de-repression is related to the amount of growth at the higher temperature.

The finding in the haploid-diploid comparisons that the rate of enzyme synthesis varies inversely with the first power of concentration of repressor forces one to conclude that, in the temperature-shift experiments, repressor does indeed disappear faster than can be accounted for by dilution alone. Thus repressor seems to be "growth-unstable" with a mean life of  $1/10$  to  $1/5$  of a generation.

It can be shown that the instability of repressor is a normal phenomenon and not simply a consequence of the temperature shifts. As will be described in section IV, the production of repressor by  $i^{TSS}$  and  $i^{s.TSS}$  strains is stabilized by the presence of inducer, full repressor levels appearing even at 42°C in  $i^{TSS}$  strains growing in the presence of  $10^{-3}$ M-IPTG. Here repressor synthesis can be arrested by removal of inducer with no change in temperature, as shown in Fig. 7. It can be seen that when inducer is suddenly removed from an  $i^{TSS}$  culture grown with inducer for many

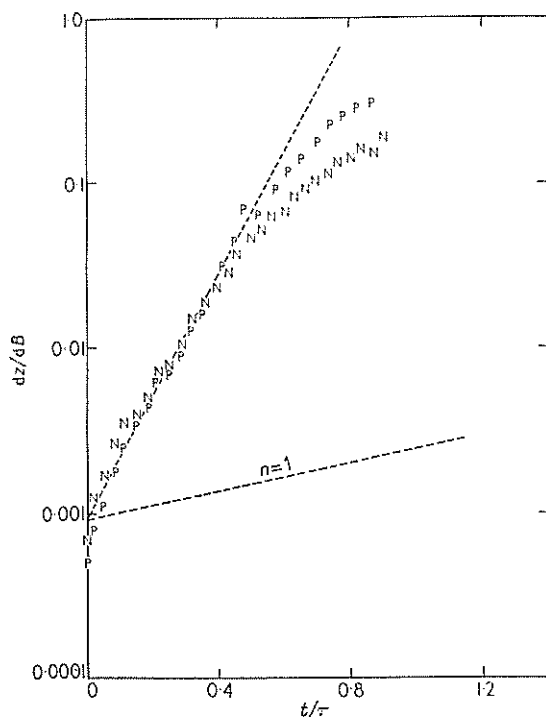


FIG. 5

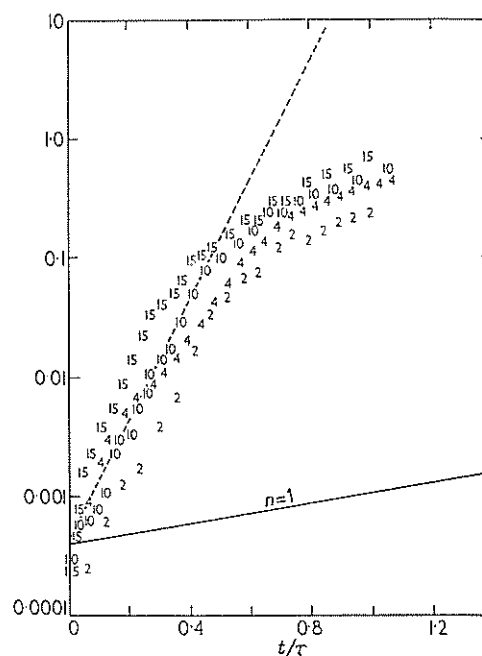


FIG. 6

FIG. 5. Kinetics of de-repression of  $\beta$ -galactosidase synthesis in E304 and E321 subsequent to a shift in growth temperature from 30 to 41.5°C.

Cultures of E304 ( $i^{u, TSSz^+y^-}$ ) and E321 ( $i^{TSSz^+y^-}$ ) which had grown for approximately 15 generations glycerol-limited in chemostats (generation times of 5 hr) at 30°C were shifted to 41.5°C at zero time. At zero time and every 10 min thereafter, samples were removed for duplicate  $\beta$ -galactosidase assays and turbidity determinations, taking care not to exceed the over-flow rate.

The rate of synthesis,  $dz/dB$ , was calculated as  $\Delta z/\Delta B$ , the ratio of the increment in  $\beta$ -galactosidase to the increment in bacterial density between adjacent measurements. The abscissa  $t/\tau$  gives bacterial growth in generations subsequent to the temperature shift. The dotted line labeled  $n = 1$  gives the expected rise in  $dz/dB$  on the assumption of a stable repressor (an  $e$ -fold decrease in repressor level for each generation of growth). The letters N and P refer respectively to E321 and E304.

FIG. 6. Kinetics of de-repression of  $\beta$ -galactosidase synthesis in E304 growing at various generation times subsequent to a shift in growth temperature from 30 to 41.5°C.

The numbers given in the Figure, "15", "10", "4" and "2" refer to the generation times (in hr) of the respective cultures. Those cultures with generation times of 15, 10 and 4 hr were growing glycerol-limited in chemostats, whereas that with a generation time of 2 hr was a batch culture. In the chemostat experiments, conditions were the same as those given for Fig. 5. Rate of synthesis,  $dz/dB$ , was calculated as in Fig. 5. The abscissa  $t/\tau$  gives bacterial growth in generations subsequent to the temperature shift. The dotted line labeled  $n = 1$  gives the expected rise in  $dz/dB$  on the assumption of a stable repressor.

generations at a high temperature, enzyme synthesis stops completely; but upon continued growth at the same temperature, de-repression occurs at a rate identical to that found in a parallel culture subjected to the temperature-shift 30 to 41.5°C. The growth instability of repressor is revealed, therefore, whether de-repression is provoked by a temperature shift or by the removal of inducer.

Moreover, the growth-instability does not seem to be unique to the repressor of  $i^{TSS}$  mutants growing at high temperatures. One piece of evidence supporting the

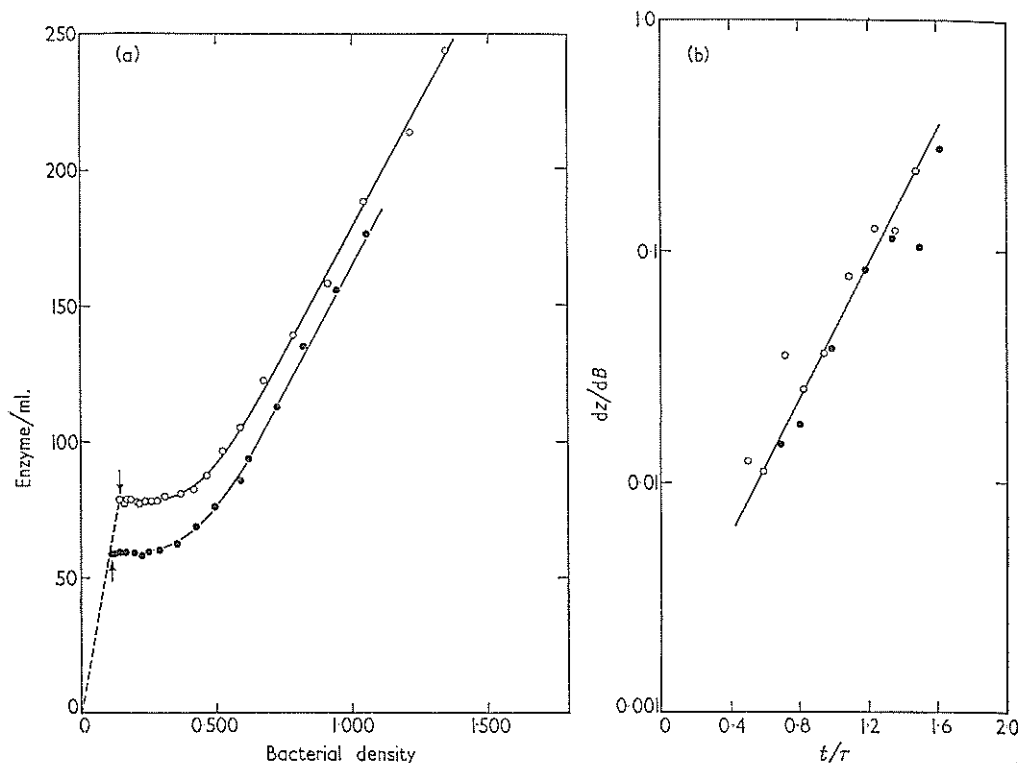


Fig. 7. (a) De-repression of  $\beta$ -galactosidase synthesis in E321 subsequent to removal of IPTG at 41°C or to a shift in growth temperature from 30 to 41°C. Parallel cultures of E321 ( $i^{TSS}z^+y^-$ ), which had grown for 12 doublings at either 30°C (—●—●—) or 41°C (—○—○—) in the presence of  $10^{-3}$  M-IPTG were filtered (at the arrows), washed and resuspended in preconditioned medium lacking IPTG at 41°C (see Materials and Methods).

(b) Kinetics of de-repression in E321 subsequent to removal of IPTG at 41°C or to a shift in growth temperature from 30 to 41°C. As in (a), (—●—●—) refers to the culture growing at 30°C prior to removal of IPTG, and (—○—○—) refers to that growing at 41°C prior to filtration. In this Figure, the results given in (a) are shown in differential form, i.e., the differential rate of  $\beta$ -galactosidase synthesis is plotted on a semi-log scale against generations of growth following the temperature shift. The line through the experimental points gives a value of  $n = 3.3$ .

hypothesis that this instability is a more general phenomenon is the fact that within the range tested (38 to 43°C) the repressor instability of  $i^{TSS}$  mutants is independent of the upper temperature used in shift-up experiments, even though the ultimate rate of enzyme synthesis varies more than tenfold in this temperature range. This suggests that instability of the repressor in  $i^{TSS}$  mutants is not reserved solely for high growth temperature, but is present—though undetectable by present means—even at low growth temperatures. A further reason to discount the possibility that instability is peculiar to the particular mutants employed comes from the fact that at lower temperatures the “basal” rate of enzyme synthesis is similar to that in the wild-type strain and not five to ten times higher in the mutant, which would be expected were the repressor unstable only in the mutant.

It should be recalled that there has been reason to believe that repressor is normally a stable molecule. In the original experiments providing evidence for the existence

of repressor (Pardee, Jacob & Monod, 1959), it was found that times of the order of a bacterial doubling were required for a newly injected  $i^+$  gene to establish repression in an initially  $i^-$  cytoplasm. Similar long delays were found in the re-establishment of repression in an  $i^{TL}$  mutant after the inactivation of repressor by pulse heating (Horiuchi & Novick, 1961). In this case the rate of enzyme synthesis falls only by a factor of ten in the generation of growth following heating. These delays could reflect the long time required for the accumulation of substantial amounts of a stable repressor, although they are unaccountably long if  $dz/dB$  varies as  $1/R$ , in which case a very small fraction of the steady-state repressor level would produce a sharp reduction in rate of enzyme synthesis. The discrepancy becomes more severe in the case of an unstable repressor, which should approach its maximum level even more quickly. Many explanations can be imagined for this paradoxical behavior, among them delays in the appearance of repressor (see section V of Results) or delays in sensitivity of the *Lac* operator to repressor. It should be noted, however, that such delays do not always occur. For example, as shown in Fig. 8, when a de-repressed  $i^{s, TSS}$  culture growing (generation time of 200 minutes) at 43°C is shifted to 30°C,

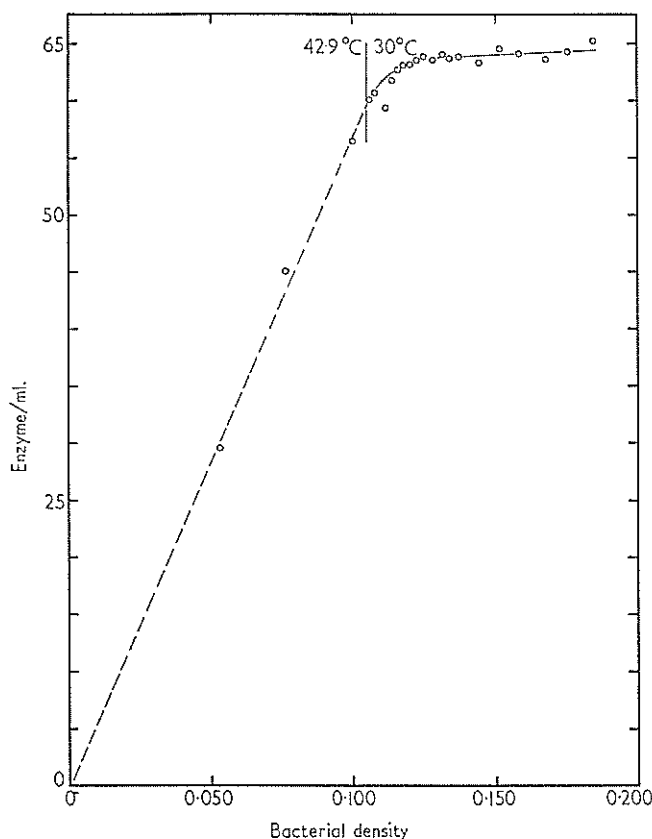


FIG. 8. Onset of repression of  $\beta$ -galactosidase synthesis in E304 following a shift in growth temperature from 42.9 to 30°C.

At the point indicated, a culture of E304 ( $i^{s, TSS} + y^-$ ) which had grown at 42.9°C for many generations was shifted to 30°C. The medium was the same as that specified for E304 in Fig. 1. Thermal equilibration of the culture was essentially complete within 3 min following the shift. Each point given in the Figure is the average of four assays.

enzyme synthesis is essentially stopped within 6% of a generation. In a similar shift-down experiment, utilizing the same strain growing carbon-limited in a chemostat with a generation time of 12 hours, no detectable increment of enzyme was synthesized after the temperature shift.

(IV) *Structural changes in repressor caused by inducer*

In the Jacob-Monod model it is proposed that the inducer brings about an allosteric transition ("a discrete reversible alteration of the molecular structure") in the repressor, thereby removing its inhibitory effect on the operator (Monod, Changeux & Jacob, 1963). That such transitions do actually occur is supported by two independent effects of inducer on the thermal properties of the repressor in the temperature-sensitive mutants.

The first effect is seen in strains having the  $i^{TL}$  heat-labile repressor. Here it was earlier found that inducer, added during heating, does not stabilize this repressor against thermal inactivation (Horiuchi & Novick, 1965). It was also observed in these studies that the repressor in  $i^{TL}$  strains is not uniformly inactivated; at a given temperature only a certain fraction of repressor can be inactivated, and this fraction increases with temperature. Furthermore, inactivation is irreversible, ruling out some kind of equilibrium as the explanation of the apparent heterogeneity. In the present work it was discovered that in the presence of inducer the heterogeneity is abolished.

The effect of inducer on the stability of  $i^{TL}$  repressor is shown in Fig. 9. It can be seen that, after heating an  $i^{TL}$  strain at 37°C for 80 minutes in the absence of inducer, the extent of de-repression (measured by the initial slope after return to growth at 25°C) reaches about 20% of maximum (further heating at 37°C gives no additional de-repression). If, however, IPTG is present only during heating, the subsequent de-repression approaches 100%, as can be seen by comparison with a culture fully induced by excess IPTG during growth. The kinetics of repressor inactivation at 37°C in the presence or absence of IPTG ( $5 \times 10^{-4}M$ ) deduced from data of the type in Fig. 9 is given in Fig. 10.

Since thermal stability of  $i^{TL}$  repressor molecules is affected by the inducer, it follows that IPTG produces some kind of structural change in the repressor.

A second demonstration of the interaction of repressor and inducer was found in experiments with  $i^{TSS}$  strains. It had been found earlier that the presence of IPTG in the concentration range above  $10^{-4}M$  in cultures of  $i^{s,TSS}$  strains at intermediate temperatures decreases the rate of production of  $\beta$ -galactosidase (Novick, Lennox & Jacob, unpublished experiments). This effect is shown in Fig. 11. That this effect is due to an increased rate of production of repressor follows from the fact that considerable time is required after addition or removal of inducer to obtain the ultimate rate of synthesis (Fig. 12). Were it the result of an enhancement of repressor effectiveness, the change in rate would be immediate.

To account for this stabilization of repressor formation in the  $i^{TSS}$  strains, one must first consider how the synthesis of a specific macromolecule can be temperature-sensitive. As suggested above, a plausible explanation is that some specific precursor is heat-labile whereas the final substance is stable. On this basis, one can suppose in the present case that the precursor has a site for IPTG and is stabilized by it, thus increasing the precursor level and the probability of its conversion to repressor. As will be argued below, it is likely that the precursor is a subunit of a polymeric repressor molecule.

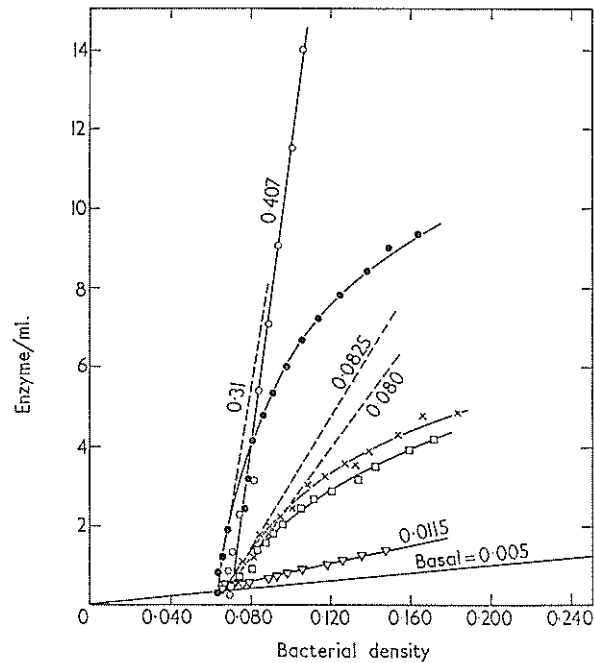


Fig. 9. Effect of IPTG on the thermolability of the  $i^{T^L}$  repressor.

E103a ( $i^{T^L}z^+y^-$ ), which had grown for approximately 10 doublings in F medium supplemented with Casamino acids (200  $\mu\text{g}/\text{ml.}$ ) and L-tryptophan (10  $\mu\text{g}/\text{ml.}$ ) at 25°C, was filtered, washed (see Materials and Methods) and resuspended in F buffer containing L-canavanine- $\text{H}_2\text{SO}_4$  (100  $\mu\text{g}/\text{ml.}$ ) and 5-methyltryptophan (100  $\mu\text{g}/\text{ml.}$ ). To one portion of this suspension IPTG was added to a final concentration of  $5 \times 10^{-4}$  M and it was incubated at 25°C ( $\nabla$ ). To another the same concentration of IPTG was added, but it was incubated at 37°C ( $\bullet$ ). Three other portions were incubated at 37°C without the addition of IPTG. At the end of the incubation period (80 min in all cases), immediately before filtration and washing, one of these ( $\times$ ) received IPTG to  $5 \times 10^{-4}$  M. All suspensions were then chilled in ice, filtered and washed with iced buffer and resuspended in preconditioned medium containing Casamino acids and tryptophan at 25°C. To one ( $\circ$ ) IPTG was immediately added to a final concentration of  $5 \times 10^{-4}$  M. Growth was immediate in all cases (generation times of 165 to 175 min). All cultures were sampled periodically for 3 hr for turbidity measurements and duplicate  $\beta$ -galactosidase assays. The dotted lines and numbers given in the Figure refer to the initial rates of synthesis ( $dz/dB$ ) following the heat pulse.

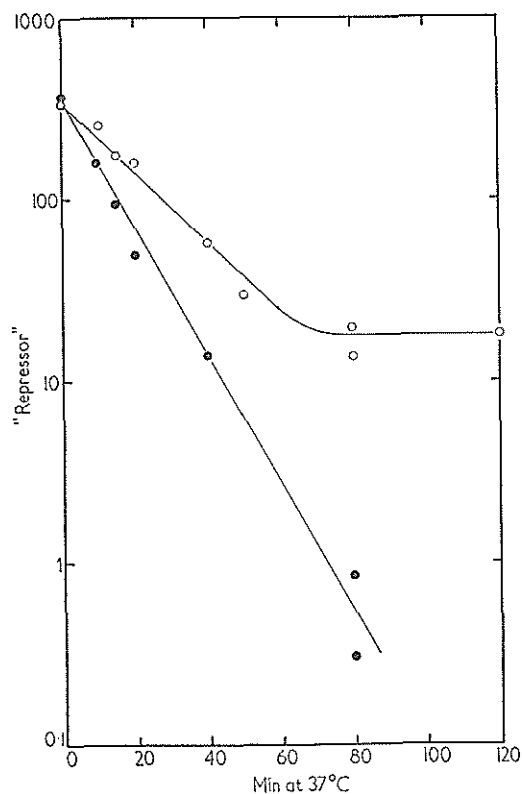


FIG. 10. Kinetics of  $\lambda^{T_L}$  repressor inactivation at 37°C in the presence or absence of IPTG.

Experimental conditions were as described in the legend of Fig. 9. E103a growing at 25°C was removed into buffer containing canavanine and 5-methyltryptophan, and heated for various periods at 37°C in the presence (—●—●—) or absence (—○—○—) of IPTG ( $5 \times 10^{-4}M$ ). After return to growth at 25°C, each culture was sampled at 10-minute intervals for assay and turbidity determinations. The repressor level was calculated from the initial rate of synthesis following the heat pulse,  $(dz/dB)_0$  from the relationship

$$\left(\frac{dz}{dB}\right)_0 = \left(\frac{dz}{dB}\right)_{\max} \frac{K}{K + R}$$

where  $(dz/dB)_{\max}$  was determined on cultures treated identically save for the fact that they received IPTG ( $5 \times 10^{-4}M$ ) during subsequent growth.



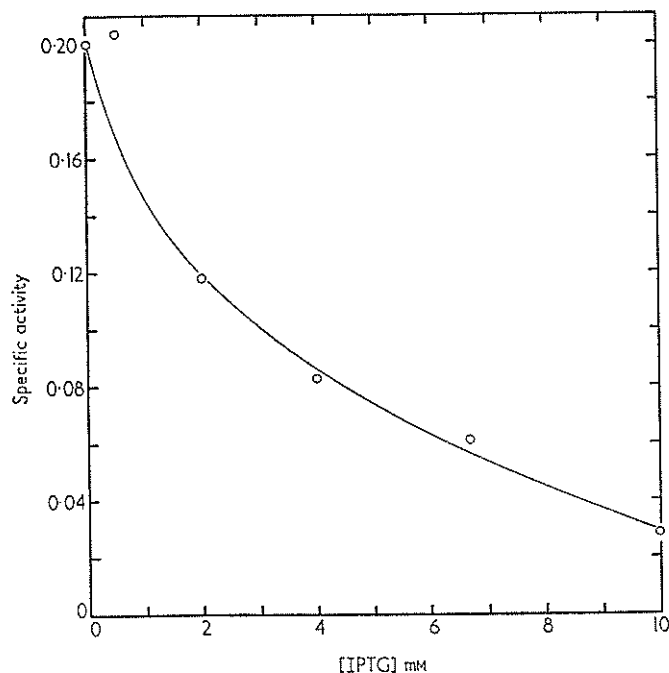


FIG. 11. Steady-state specific activities of  $\beta$ -galactosidase in E304 at 38.5°C in the presence of various concentrations of IPTG.

E304 was grown for a minimum of 10 doublings in IPTG at the specified concentrations at 38.5°C prior to sampling for assay. The medium used was that specified for E304 in Fig. 1.

It might seem surprising to find this apparent interaction of a repressor precursor and IPTG in the case of  $i^{s.TSS}$  strains, since the repressor in these strains when present is of the  $i^s$  type, i.e. IPTG produces no induction of these strains. However, such strains do begin to be induced by exceedingly large ( $10^{-1}M$ ) concentrations of IPTG (Cohn, personal communication). If the rate of enzyme synthesis falls as  $1/R$ , in order to produce the thousandfold increase in rate of enzyme synthesis normally seen upon induction, concentrations of inducer leading to a thousandfold fall in effective concentration of repressor must be employed. To cause a tenfold increase in concentration of repressor, much lower concentrations of inducer should suffice, since such an increase represents a stabilization of only 1/100 of the total repressor being made.

One would expect to see the same kind of stabilization of production of  $i^{TSS}$  repressor by inducer, but the effect should occur at much lower concentrations of IPTG since strains having this repressor are inducible by much lower concentrations of IPTG. That this is the case can be seen in Fig. 13, where the specific activity of cultures grown for many generations at three temperatures at a series of concentrations of IPTG are plotted. It can be seen that at lower concentrations of IPTG there is an inhibitory effect on synthesis of enzyme, attributable to an increased level of repressor. Evidently, at these concentrations of IPTG the increase in repressor exceeds the inducing effect. As expected, at high concentrations of IPTG the bacteria become fully induced, and the expected increase in repressor level is not directly observable.

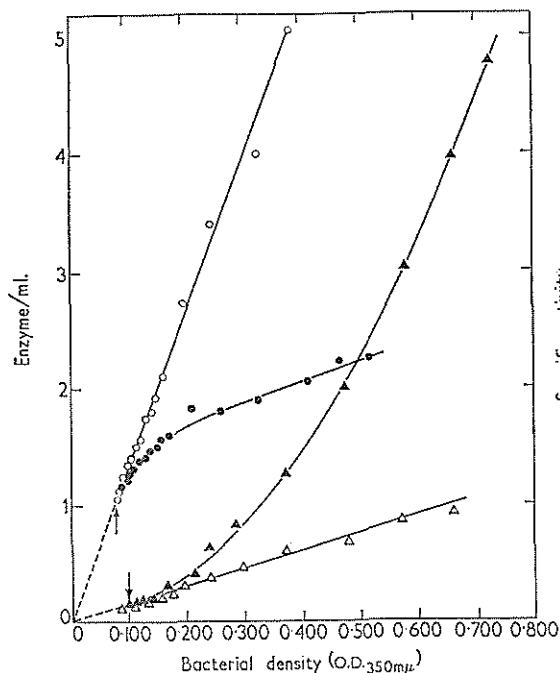


FIG. 12

FIG. 12. Onset of repression or de-repression in E304 at 38°C following the addition or removal of 0.01 M-IPTG.

Parallel cultures of E304 ( $i^s, T^{ss}z^+y^-$ ), which had grown in the presence ( $\Delta$ ,  $\blacktriangle$ ) or absence ( $\circ$ ,  $\bullet$ ) of 0.01 M-IPTG for at least 10 doublings at 38°C, were at the point indicated (arrows) filtered, washed and resuspended in preconditioned medium. The culture which had previously grown in IPTG was divided into two parts, one part ( $\Delta$ — $\Delta$ — $\Delta$ ) receiving IPTG at the same concentration, the other ( $\blacktriangle$ — $\blacktriangle$ — $\blacktriangle$ ) none. Likewise the culture which had previously grown in the absence of IPTG was divided into two parts, one receiving IPTG to a final concentration of 0.01 M ( $\bullet$ — $\bullet$ — $\bullet$ ), the other ( $\circ$ — $\circ$ — $\circ$ ) none. Samples were removed for assay by turbidity determinations every 15 min initially and thereafter every 30 min. The medium used was that specified for E304 in Fig. 1.

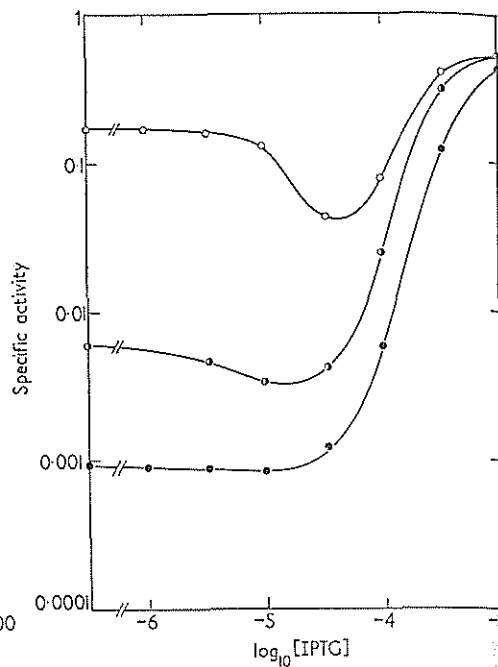


FIG. 13

FIG. 13. Effect of various concentrations of IPTG on the steady-state levels of  $\beta$ -galactosidase in E321 growing at three temperatures.

Cultures of E321 ( $i^{T^{ss}z^+y^-}$ ) were grown in the specified concentrations of IPTG for 10 to 15 doublings at the temperatures 29.8°C ( $\bullet$ — $\bullet$ — $\bullet$ ), 36.2°C ( $\circ$ — $\circ$ — $\circ$ ) and 40°C ( $\Delta$ — $\Delta$ — $\Delta$ ) prior to sampling for assay. Each point given is the average of two or more assays on each of two parallel cultures.

Another demonstration of the stabilization of repressor formation is seen in Fig. 7(a). Here bacteria grown for many generations at 42°C in the presence of a high concentration of IPTG were removed from IPTG and grown further at 42°C. The presence of repressor is indicated by the immediate cessation of enzyme synthesis following removal of inducer, a synthesis which is resumed as the bacteria become de-repressed upon further growth. The behavior is similar to that of an  $i^{T^{ss}}$  culture transferred from 30 to 42°C, and it thus appears that at 42°C in the presence of high concentration of IPTG the bacteria have nearly maximal levels of repressor.

If bacteria growing at 42°C are exposed to IPTG for three minutes, one would expect a production of about 2% of the full repressor level (generation time = 150 minutes). Furthermore, if, as concluded above,  $dz/dB$  varies as  $K/(K+R)$ , where  $R_{\max} = 1000K$ , 20  $K$  units of repressor would be formed, leading to a sharp drop in rate of synthesis

of  $\beta$ -galactosidase. This expectation has been confirmed in a number of experiments in which three-minute pulses of IPTG ( $10^{-3}$  M) produce tenfold decreases in  $dz/dB$  in  $i^{TSS}$  strains growing at 41 to 42°C.

A more quantitative demonstration of the stabilizing effect of IPTG on repressor formation in  $i^{TSS}$  strains is presented in Fig. 14. Here the initial rate of synthesis was observed following removal of IPTG for a series of cultures grown for many generations at various concentrations of IPTG. When the results in Fig. 14 are compared with those in Fig. 11, it can be seen that the apparent  $K_m$  for IPTG is about 200 times less in the case of the  $i^{TSS}$  repressor than in the case of  $i^{s,TSS}$  repressor. This is about the same ratio as that observed in the concentrations of IPTG required for induction of enzyme formation in the two cases, in agreement with the belief that the same interaction of inducer and repressor is involved in both induction and thermal stabilization.

#### (V) *The possible polymeric nature of repressor*

A plausible explanation, as noted in section I, for the fact that synthesis of repressor is temperature-sensitive in  $i^{TSS}$  strains is that repressor is a polymer, itself thermostable, but assembled from subunits (monomers) which are thermolabile. This interpretation is favored because of the fact that synthesis of repressor is stabilized by inducer, a finding which can be explained by the assumption that the monomer has a site for inducer and that combination with inducer stabilizes it against thermal inactivation.

Another indication that the repressor may be polymeric comes from the fact that in  $i^+z^+y^-$  strains the extent of induction rises with the square of inducer concentration (Boezi & Cowie, 1961); this is illustrated in Fig. 15. From the conclusions here that  $dz/dB$  varies as  $1/R$  and that induction is equivalent to inactivation of repressor, it seems that two molecules of inducer are required to inactivate a repressor molecule, an indication of at least two inducer sites per repressor molecule.

These interpretations of the structure and mechanism of synthesis of repressor lead to the expectation that modest increases in precursor level would cause disproportionately large increases in repressor level in  $i^{TSS}$  strains at those temperatures where the precursor is significantly thermolabile. To test this expectation, *F-Lac* homogenotes of E304 ( $i^{s,TSS}z^+y^-$ ) and E321 ( $i^{TSS}z^+y^-$ ) were prepared and their steady-state differential rates of  $\beta$ -galactosidase synthesis were compared with those of the respective haploid strains. The results, given in Figs 16 and 17, are in good agreement with this expectation. In the temperature range 36 to 38°C, where the effect is maximal, the *F-Lac* homogenotes of E304 and E321 synthesize  $\beta$ -galactosidase at rates only 1/8 and 1/17 of the respective haploid parents. At low growth temperature the ratio diploid/haploid approaches unity, which is to be expected if the monomeric species is stable and if the rate of synthesis is determined in each case by the level of repressor. At the highest growth temperatures or under conditions of maximal induction, the *F-Lac* homogenotes have rates of synthesis 2.0 to 2.5 times those of the respective haploids, confirming the hypothesis of higher ploidy of the *F*-genotes. In distinction from the marked difference in thermal de-repression between  $i^{TSS}z^+y^-$  and  $i^{s,TSS}z^+y^-/F i^{TSS}z^+y^-$  at a given temperature, under conditions of induced synthesis at low growth temperatures the two strains behave like the corresponding  $i^+$  strains (Fig. 4), indicating that the relationship between rate of synthesis and repressor level is normal in these strains.

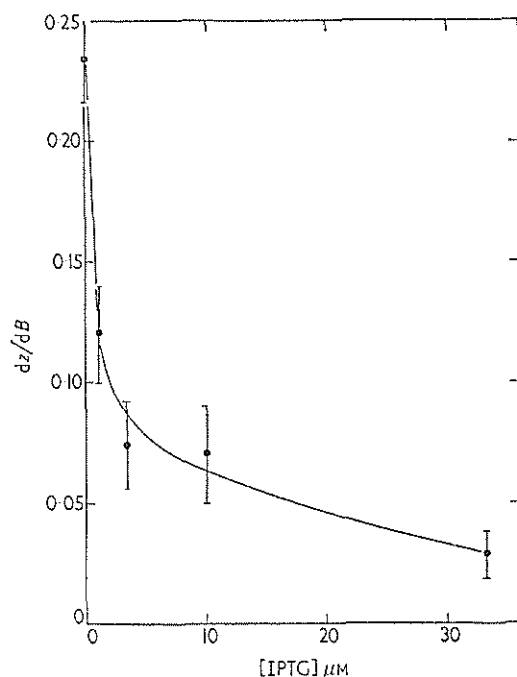


FIG. 14

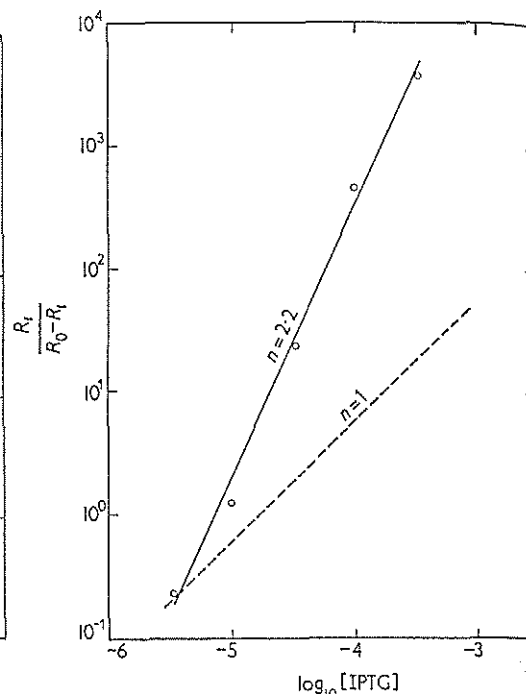


FIG. 15

FIG. 14. Stabilization of repressor in E321 growing at 41.5°C by various concentrations of IPTG.

E321 ( $i^{TSSz^+y^-}$ ) was grown for 10 doublings or more at 41.5°C in the presence of IPTG at the concentrations specified. IPTG was then removed by filtration and washing of the cells, always at 40°C. The cells were then resuspended in preconditioned medium at 41.5°C, and samples for assay in quadruplicate and turbidity determinations were removed at 5-min intervals. Growth was immediate in all cases. The initial differential rates of synthesis ( $dz/dB$ ) following removal of IPTG shown in the Figure were determined from enzyme/ml. ( $z$ ) versus turbidity ( $B$ ) plots in each case. The error bar for each point indicates the uncertainty in the initial rate of synthesis.

FIG. 15. Stoichiometry of induction with IPTG in strain W14.

Experimental conditions were as given for W14 in Fig. 4. The level of active repressor for each concentration of IPTG was calculated on the basis of the equation  $\frac{dz}{dB} = \frac{K}{K + R} \left( \frac{dz}{dB} \right)_{\max}$ . The total repressor level  $R_0$  was calculated from the same equation using the basal rate of enzyme production (about 0.1% of the fully induced rate).  $\left( \frac{dz}{dB} \right)_{\max}$  was assumed to be equal to the steady-state rate in the presence of  $10^{-3}$  M-IPTG. The ordinate,  $R_i/(R_0 - R_i)$ , gives the ratio of inactive repressor to active repressor at each concentration of IPTG. The dotted line  $n = 1$  gives the expected slope on the assumption that one molecule of IPTG is sufficient to inactivate one molecule of repressor.

Since it is unlikely that the TSS mutations alter the basic mechanism of repression, the lower rate of synthesis in the TSS diploids indicates a disproportionate increase in level of repressor (by a factor of 16 to 34 rather than 2.5) in these diploids. Such disproportionate increases in repressor level in these cases can be explained, as anticipated above, by the assumption that the repressor is a polymer and that in the TSS strains the monomeric precursors are thermolabile. The results are consistent with the hypothesis that the number of such subunits is about four.

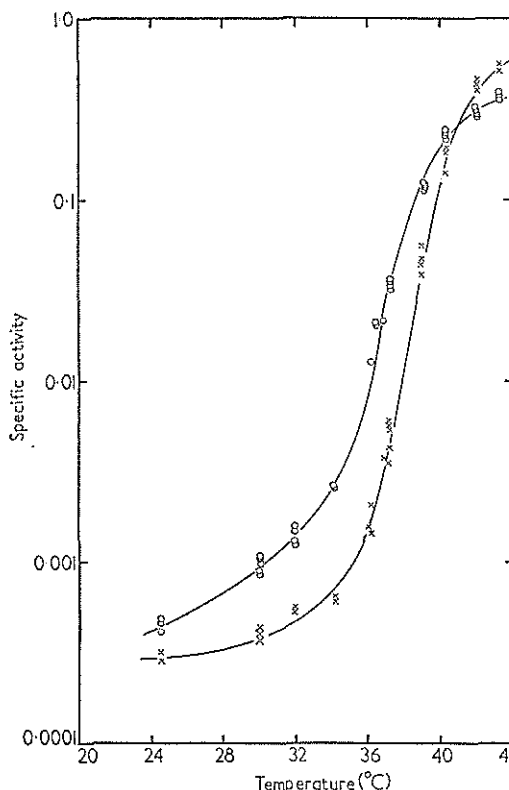


FIG. 16

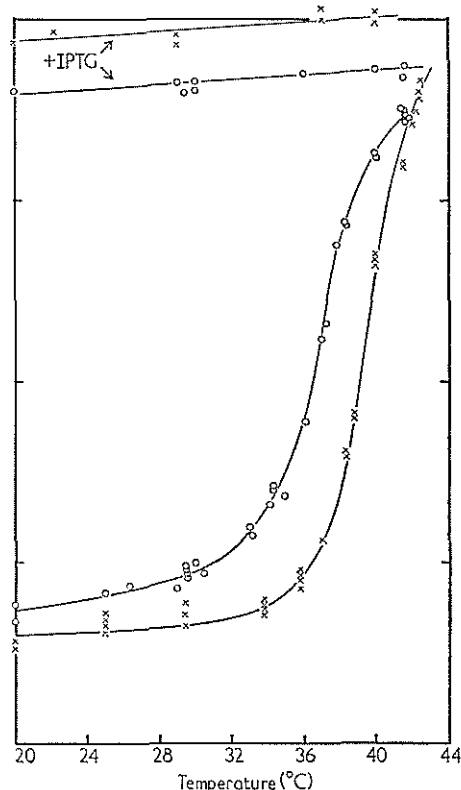


FIG. 17

FIG. 16. Steady-state levels of  $\beta$ -galactosidase in E304 and E310 at various growth temperatures. E304 ( $i^s, TSS_z^+ y^-$ ) and E310 ( $i^s, TSS_z^+ y^- / F_{i^s, TSS_z^+ y^-}$ ) were grown at the specified temperature for 10 or more doublings prior to enzyme assay. In all cases both the haploid and the  $F$ - $Lac$  homogenote were grown in the same shaking water bath to insure identical thermal histories of the two strains. The medium used was that specified for E304 in Fig. 1. Each experimental point for haploid ( $\circ$ ) and  $F$ - $Lac$  homogenote ( $\times$ ) is the average of two assays on the same culture at the same time.

FIG. 17. Steady-state levels of  $\beta$ -galactosidase in E321 and E323 growing at various temperatures in the presence or absence of  $10^{-3}M$ -IPTG.

Experimental conditions were the same as those given for Fig. 16. The medium used was that specified for E321 in Fig. 1. In the upper curves IPTG was present in the medium at a final concentration of  $10^{-3}M$ . Each experimental point for the haploid ( $\circ$ ) E321 ( $i^{TSS_z^+ y^-}$ ) and the  $F$ - $Lac$  homogenote ( $\times$ ) E323 ( $i^{TSS_z^+ y^-} / F_{i^{TSS_z^+ y^-}}$ ) is the average of two assays on the same culture at the same time.

#### 4. Discussion

In the present studies, the  $i$ -gene product is identified more clearly as the postulated repressor of  $\beta$ -galactosidase synthesis. Furthermore, it is shown that the rate of synthesis of  $\beta$ -galactosidase varies reciprocally with the first power of level of repressor, refuting the earlier inference of a high power dependence. A similar dependence has recently been reported for alkaline phosphatase synthesis (Gallant & Spottswood, 1965). The assumption has been made here that the rate of  $\beta$ -galactosidase synthesis is defined uniquely by the level of repressor, other things being equal.

The fact that consistent results are obtained in experiments where repressor is varied by growth temperature in  $i^{TL}$  strains and by varying inducer with  $i^+$  and  $i^{TL}$  strains supports this assumption and its corollary, that induction is no more than (reversible) inactivation of repressor.

The present results also offer direct evidence for an interaction between inducer and repressor. Such an interaction had been postulated earlier (Monod *et al.*, 1963), and evidence for this first came from the discovery of mutations in the  $i$ -gene that lead to an altered specificity toward inducers (Willson *et al.*, 1964). In the present work, from the changes in thermal behavior produced by inducer in the various temperature mutants, it is concluded that the interaction of inducer and repressor leads to a structural alteration in repressor. Such a change in structure in response to the presence of a small molecule like the inducers used here makes it likely that repressor is indeed an allosteric protein, as argued earlier (Monod *et al.*, 1963). The evidence even seems to indicate that repressor is a polymeric protein, as proposed in the allostery model, although there is no evidence in the present studies that the state of polymerization is affected by inducer. It should be emphasized that these considerations require only that repressor be a polymer of units defined in the  $F$ - $Lac$  episome, and do not necessarily indicate a homopolymer.

What is unexpected is the conclusion that repressor is unstable under conditions of bacterial growth, although such a finding is not inconsistent with the model of Jacob & Monod. A similar instability can be inferred for  $\lambda$  phage immunity substance from studies of a  $\lambda$  mutant in which prophage induction occurs at higher temperatures but only after growth (Sussman & Jacob, 1962). The fact that disappearance of repressor is closely coupled to growth argues against its being simply an unstable protein. To account for the growth dependence, one could imagine that the repressor is formed through the association of the  $i$ -gene product (a protein) with another constituent that is growth-unstable. The most obvious candidate for the latter is m-RNA, although the mean life of repressor is ten to thirty times that usually found for m-RNA (which could result, however, from the association of an m-RNA with a specific protein). Growth instability could also result if the  $i$ -gene product were unstable with respect to dilution, as is the case for a polymer in equilibrium with its monomeric elements.

Alternatively, it is possible that the disappearance of repressor is a consequence of the act of repression. For example, if repressor could become attached to the  $Lac$  m-RNA, thus blocking ribosome attachment and subsequent translation into enzymes, the kinetics of de-repression observed after shifting to high temperature would correspond to the depletion of the repressor pool through m-RNA synthesis. One would have to imagine that blocking of translation would have the secondary effect of slowing DNA transcription. In objection, there is evidence that repressor does not interfere with translation (Kepes, 1963; Nakada & Magasanik, 1964).

Many points thus remain unsettled. Although the evidence favors the hypothesis that repressor is an allosteric protein, no satisfactory explanation for its growth instability is at hand. Moreover, although it is clear that repressor does interact with inducer, it is not yet settled whether it blocks m-RNA production by a direct action on a DNA operator gene or by an indirect action involving the m-RNA itself.

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